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Role of Deoxyribose Catabolism in Colonization of the Murine Intestine by Pathogenic *Escherichia coli* Strains▽

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We previously suggested that the ability to metabolize deoxyribose, a phenotype encoded by the *deoK* operon, is associated with the pathogenic potential of *Escherichia coli* strains. Carbohydrate metabolism is thought to provide the nutritional support required for *E. coli* to colonize the intestine. We therefore investigated the role of deoxyribose catabolism in the colonization of the gut, which acts as a reservoir, by pathogenic *E. coli* strains. Molecular and biochemical characterization of 1,221 *E. coli* clones from various collections showed this biochemical trait to be common in the *E. coli* species (33.6%). However, multivariate analysis evidenced a higher prevalence of sugar-metabolizing *E. coli* clones in the stools of patients from countries in which intestinal diseases are endemic. Diarrhea processes frequently involve the destruction of intestinal epithelia, so it is plausible that such clones may be positively selected for in intestines containing abundant DNA, and consequently deoxyribose. Statistical analysis also indicated that symptomatic clinical disorders and the presence of virulence factors specific to extraintestinal pathogenic *E. coli* were significantly associated with an increased risk of biological samples and clones testing positive for deoxyribose. Using the streptomycin-treated-mouse model of intestinal colonization, we demonstrated the involvement of the *deoK* operon in gut colonization by two pathogenic isolates (one enteroaggregative and one uropathogenic strain). These results, indicating that deoxyribose availability promotes pathogenic *E. coli* growth during host colonization, suggest that the acquisition of this trait may be an evolutionary step enabling these pathogens to colonize and persist in the mammalian intestine.

Escherichia coli is a normal inhabitant of the intestines of healthy individuals. However, under certain circumstances, *E. coli* can also be a serious pathogen in humans and animals. Many studies over the last 30 years have focused on the identification and description of numerous virulence factors and the genes encoding them. There is now a substantial body of knowledge concerning the development of three major clinical syndromes caused by these bacteria: urinary tract infections (UTI), sepsis/meningitis, and diarrhea. The strains causing the extraintestinal diseases are referred to as ExPEC (*ex*traintestinal *p*athogenic *E. coli*) strains and include uropathogenic *E. coli* and sepsis/meningitis-associated *E. coli* strains. The intestinal pathogens are distributed into at least six well-described pathovars—enteropathogenic *E. coli*, Shiga toxin-producing *E. coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli*—which exhibit differing physiopathological behavior patterns (33, 41). The reservoir for all pathogenic *E. coli* strains is the gut, and it remains unclear how pathogenic *E. coli* strains outcompete the gut microflora to colonize the mammalian intestine and how they survive in this complex ecosystem. Increasing our understanding of these two issues may facilitate the development of new therapeutic strategies against these highly prevalent infections.

There is diverse evidence that *E. coli* strains isolated from the feces of healthy people use nutrients derived from the mucus for their intestinal growth and that monosaccharide catabolism plays a key role in mouse intestine colonization (47, 9). Comparative genomic analyses of *E. coli* strains indicate that many genomic islands specific to pathogenic isolates harbor genes encoding proteins with metabolic functions, including the transport and utilization of carbohydrates (35, 12, 8). However, little is known about the relationship between virulence and the functionality of these regions.

The ability of various *E. coli* strains to degrade deoxyribose

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is long established and has been used in biotyping assays to discriminate between clinical isolates (32, 31, 15). However, it was only recently that the association between this metabolic trait and the pathogenic potential of the strain has been demonstrated (35, 5). We showed that deoxyribose use in *E. coli* depends on proteins encoded by the *deoK* operon and that this operon was acquired by horizontal transfer from *Salmonella enterica* (5). Deoxyribose catabolism is clearly dependent on deoxyribokinase (the product of the *deoK* gene), which catalyzes the ATP-dependent phosphorylation of 2-D-deoxyribose to generate 2-D-deoxyribose-5-phosphate, a key product of the deoxynucleoside catabolism pathway (5, 45). A putative permease (DeoP), a mutarotase (DeoM), and a transcriptional regulator (DeoQ) are also involved in this function (3, 13, 45). We assessed the advantage conferred by expression of the *deoK* operon through in vitro coculture experiments and found that deoxyribokinase activity conferred a late-stationary-phase growth advantage on the strain (5).

Here, we report an exploration of the role in host colonization of sugar catabolism by pathogenic *E. coli* strains. First, we studied deoxyribose use by *E. coli* strains. We assessed the prevalence of this biochemical characteristic at the species level by analyzing strains from the ECOR collection, representative of the diversity of the species; we also determined its prevalence in a collection of 1,149 pathogenic and nonpathogenic strains isolated from various clinical specimens. We performed a multistate study of these human isolates that allowed us to consider deoxyribose catabolism as a "metabolic-virulence" trait. We then carried out in vivo competition experiments in the streptomycin-treated-mouse model of intestine colonization to investigate the role of deoxyribose metabolism in intestine colonization. We showed that both the diarrhea-associated enteroaggregative 55989 isolate and the uropathogenic AL511 isolate outcompeted their respective isogenic *deoK* mutants in vivo.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The EAEC strain 55989 was originally isolated from the stools of a human immunodeficiency virus-positive adult suffering from persistent aqueous diarrhea in the Central African Republic (40). This strain carries the aggregative type III adhesion fimbriae (4) and is highly lethal in a mouse model used for evaluating the intrinsic virulence of *E. coli* strains (E. Denamur, personal communication). The strain is also resistant to tetracycline. The uropathogenic *E. coli* strain AL511 was isolated from the urine of a woman suffering from pyelonephritis in France (2). It carries and expresses genes encoding virulence factors specific to ExPEC (P fimbriae and F17 and AfaE-VIII adhesins) (24) (C. Pichon, personal communication). In a mouse model of ascending pyelonephritis, the interaction of this strain with renal collecting duct epithelial cells stimulates an innate immune response (10). The strain is resistant to ampicillin, chloramphenicol, spectinomycin, streptomycin, and tetracycline. Several collections of *E. coli* strains were also used in this study. The ECOR collection was provided by the Biological Resources Center of Institut Pasteur. It consists of 72 strains isolated from different continents and from both human and animal hosts (43). We also used a collection of strains isolated between 1999 and 2002 in Romania and between 2001 and 2002 in Senegal and the Central African Republic (CAR). These isolates were obtained from specimens collected from 213 subjects with various clinical conditions. We investigated 115 urine samples from patients with UTI diagnosed (as pyelonephritis, cystitis, and asymptomatic bacteriuria) according to clinical symptoms and laboratory investigations. Ninety-eight stool samples were obtained from healthy subjects with no diagnosed infection who were not receiving treatment for infection in the days preceding recruitment and from patients with diagnosed clinical symptoms of diarrhea. *E. coli* was obtained from diarrheic stool samples as an almost pure culture (>90%) in nonselective, solid, bromocresol purple medium. No *Salmonella* spp. and no *Shigella* spp. were detected. Lactose-positive colonies were randomly picked from each stool sample and biochemically identified as *E. coli*. The colonies were also tested by PCR for the presence of genes encoding virulence factors defining the six pathotypes of diarrheagenic *E. coli*: heat-labile and heat-stable enterotoxins characteristic of enterotoxigenic *E. coli*, intimin and BFP (bundle-forming pilus) characteristic of enteropathogenic *E. coli*, IpaH (invasion plasmid antigen H) characteristic of enteroinvasive *E. coli*, Shiga-like toxins (StxI and StxII) characteristic of Shiga toxin-producing *E. coli*, plasmid pAA characteristic of EAEC, and Afa adhesins characteristic of diffusely adherent *E. coli* (23, 22). Written, informed consent was obtained from participants in Romania, Senegal, and the CAR, and local ad hoc ethics committees approved all aspects of the study.

Strains were routinely cultured in Luria-Bertani (LB) broth or on LB agar plates at 37°C. Antibiotics were used as required at the following concentrations: kanamycin, 100 mg/liter; zeocin, 60 mg/liter; apramycin, 30 mg/liter; and carbenicillin, 100 mg/liter.

Definition of deoxyribose-positive samples. Bacterial growth was tested on K5 minimal medium (18) supplemented with 1 μg/ml thiamine and 0.1% (vol/vol) deoxyribose (2-deoxy-D-ribose; Sigma) as the sole source of carbon. Appropriate agar plates were inoculated with a loop of bacteria grown on LB agar, washed twice, and resuspended in sterile water. The plates were incubated at 37°C for 24 to 48 h. Isolates were screened for the *deoK* operon by colony hybridization as described by Grunstein and Hogness (26) under stringent (65°C) conditions with a probe labeled with [^{32}P]dCTP using the Megaprime labeling system (Amersham). The probe used was an internal fragment (831 bp) of the *deoK* gene amplified with the following primers: deoK-F (5′-ATCAGATGCCTAAAGAAGGAGAAAC-3′) and deoK-R (5′-CAATACTCGGATAAGATGATTGC-3′). *E. coli* strains AL862 (35) and HB101 (7) were used as positive and negative controls, respectively, for the presence and expression of the *deoK* operon. A clone was considered deoxyribose positive if the *deoK* gene was detected by PCR and it used the sugar.

Strain construction. A spontaneous streptomycin-resistant derivative of strain 55989 was selected. Mutants of the 55989Str and AL511 strains with deletions in genes involved in metabolic pathways were generated with the lambda Red recombinase system of Datsenko and Wanner (16) or a derived three-step method as previously described by Chaveroche et al. (11) and detailed at http://www.pasteur.fr/recherche/unites/Ggb/3SPCRprotocol.html. Strain constructions are detailed in Table 1, and the primers used are listed in Table 2. All allelic exchanges were verified by PCR with primers flanking the gene of interest (Table 2). Allelic exchanges resulted in metabolic mutants with growth defect phenotypes as assessed on solid minimal medium containing the metabolite of interest (0.1% deoxyribose, 0.2% gluconate [D-gluconic acid; Sigma], and 0.4% glycerol for the *deoK*, *edd*, and *glpK* mutations, respectively).

Murine model of intestinal colonization. The competitive murine model was used to investigate intestinal colonization as previously described (39), except that BALB/c mice (Charles River Laboratories, France) were used. Briefly, the mice were given drinking water containing 5 g/liter streptomycin sulfate to reduce the count of facultative resident bacteria (39). No *E. coli*-like colonies were recovered before challenge from the feces of the mice used in this study. After 1 day of treatment and overnight deprivation of food and water, the mice were fed bacteria in 200 μl of sterile 20% sucrose and 2.6% (wt/vol) sodium carbonate, pH 8. For the 55989Str and AL511 strains, 10^5 and 10^7 CFU, respectively, were used to infect mice. In coinfection challenges, the ratio between the two strains was 1:1. The mice were then returned to their normal diet, including water containing streptomycin, and were housed individually. In the following days, freshly collected fecal samples were weighed, homogenized, diluted in phosphate-buffered saline (1×), and plated on LB agar plates with and without kanamycin to differentiate between the two strains. The plates were incubated for 18 h at 37°C, and colonies were then counted. The detection limit for fecal plate counts was 10^2 CFU/g of feces. The numbers of *E. coli* mutants were determined directly from the kanamycin-containing plates. Parental counts were obtained by subtracting the number of CFU on kanamycin-selective plates from that on LB plates without antibiotic. One hundred colonies were transferred with a toothpick from plates without antibiotic to plates with or without kanamycin to check these counts. The fraction of the total count sensitive to kanamycin was determined. The numbers of colonies of the parental 55989Str and AL511 strains obtained with these two methods were highly consistent.

Colonizing ability was assessed by determining strain persistence in feces (log CFU/g of feces). The competitive index (CI) is an alternative measure evaluating the degree of virulence attenuation due to a given mutation in coinfections (6); it was calculated as the ratio of kanamycin-resistant derivative colonies to parental-strain colonies recovered from feces divided by the ratio of the corresponding strains in the inoculum. The results are expressed as log CI in the

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source
E. coli strains		
55989	Enteroaggregative strain producing AAF-III fimbriae	40
55989Str	Spontaneous streptomycin-resistant derivative of 55989	This study
55989Str *deoK*	55989Str isogenic mutant with the *deoK* gene replaced by the kanamycin cassette from MC4100*ybeW*::GB	This study
55989Str *edd*	55989Str isogenic mutant with the *edd* gene replaced by the kanamycin cassette from MC4100*ybeW*::GB	This study
55989Str *glpK*	55989Str isogenic mutant with the *glpK* gene replaced by the kanamycin cassette from MC4100*ybeW*::GB	This study
AL511	Isolate from urine of a patient with acute pyelonephritis	2
AL511 *deoK*	AL511 isogenic mutant with the *deoK* gene replaced by the FRT-flanked kanamycin cassette from pKD4	This study
AL511 *deoK* (Km^s)	AL511 *deoK* with the FRT-flanked kanamycin cassette deleted	This study
MC4100*ybeW*::GB	Strain carrying a kanamycin cassette for red recombinase-mediated knockout	J.M. Ghigo
Plasmids		
pKOBEGA	Red recombinase expression plasmid; ampicillin resistant	J.M. Ghigo
pKOBAPRA	Red recombinase expression plasmid; apramycin resistant	J.M. Ghigo
pCP20.Zeo	Flipase (Flp) expression plasmid for removal of resistance cassette; zeocin resistant	C. Pichon
pKD4	FRT-flanked kanamycin cassette template for red recombinase-mediated knockout; kanamycin resistant	16
pZEZeo*GFP*	Zeocin cassette inserted in place of kanamycin cassette in pZEKm*GFP* (J. M. Ghigo); zeocin resistant	This study
pZEZeo*deoK*	*deoK* gene from AL511 inserted in place of *GFP* in pZEZeo*GFP*; zeocin resistant	This study

figures. A log CI of zero indicated that the two strains were recovered in the same ratio as that in which they were inoculated; negative values indicated that the parental strain outcompeted the kanamycin-resistant mutant, and positive values indicated that the kanamycin-resistant mutant outcompeted the parental strain. Mutants were considered attenuated if the log CI was less than −0.3 (34).

Deoxyribose concentration in mouse cecal contents. Mouse cecal contents were isolated as previously described (14). Briefly, after a day of treatment with streptomycin and overnight deprivation of food and water, the mouse was sacrificed by CO_2 asphyxiation, and its cecum was removed and rinsed in HEPES-Hanks' buffer to eliminate feces. The cecal-content fraction corresponds to the pooled eluate from subsequent washes that were repeated until the eluate appeared to be free of matter and debris. Eluates (3 ml) were filtered and injected on a CarboPac MA1 4 by 250 column (Dionex Corp.) fitted to a high-pH anion-exchange chromatography (HPAEC) apparatus (Dionex Corp.). Free monosaccharides were separated using an NaOH gradient in water (0 to 0.8 M in 50 min) and quantified by measuring the area of each peak after pulsed

TABLE 2. Primer sequences

Specificity	Primer	Sequence (5′ to 3′)
Primers used for gene inactivation in *E. coli* strain 55989Str[a]		
deoK	deoK.Km.500-5	AGCCACCCAGTAGGGTCAGTGGCAGAGG
	deoK.Km.L-3	gattttgagacacaacgtggctttCCATTTCACTACCTCTTATAAACTTTCG
	deoK.Km.L-5	cttcacgaggcagacctcagcgccTAATCATCCTGCACAGTTAAAG
	deoK.Km.500-3	CAAGGAAACTCAGGCCAATCGC
glpK	glpK.Km.F	ATGACTGAAAAAAAATATATCGTTGCGCTCGACCAGGGCAaaagccacgt tgtgtctcaaaatc
	glpK.Km.R	TTACTCGTCGTGTTCTTCCCACGCCATCGCGCGTTTGACCggcgctgaggtct gcctcgtgaag
edd	edd.Km.F	ATGAATCCACAATTGTTACGCGTAACAAATCGAATCATTGaaagccacgtt gtgtctcaaaatc
	edd.Km.R	TTAAAAAGTGATACAGGTTGCGCCCTGTTCGGCACCGGACggcgctgagg tctgcctcgtgaag
Primers used for gene inactivation in *E. coli* strain AL511[b]		
deoK	deoK.KmFRT.F	ATGGACATTGCAGTTATTGGTTCCAATATGGTGGATCTCAgtgtaggctgg agctgcttc
	deoK.KmFRT.R	TTATTCGTTTAAGGTTAAAAATTCATTAAATTGTTCAATACcatatgaatat cctcctta
Primers used to check gene inactivation in *E. coli* strains 55989Str and AL511		
deoK	deoK.Ext5	GGTATCTCCTGGCCATTATC
	deoK.Ext3	CGGAGAAAACCAGATATTTGC
glpK	glpK.Ext5	tgtaggtgcatttgcctacc
	glpK.Ext3	gtgggattattgatgtgtgc
edd	edd.Ext5	taccgtaacatgatcttgc
	edd.Ext3	gtggtcaggattgattctgc

[a] Primers were designed based on the *E. coli* 55989 genome sequence (Coliscope Consortium [http://www.genoscope.cns.fr/spip/Escherichia-fergusonii,434.html]). Sequences complementary to the kanamycin resistance cassette in the template strain MC4100*ybeW*::GB are shown in lowercase letters.

[b] Primers were designed based on the *E. coli* AL511 partial genome sequence (C. Bouchier, personal communication). Sequences complementary to the kanamycin-FRT resistance cassette in the template plasmid pKD4 (16) are shown in lowercase letters.

electrochemical detection. Monosaccharides were identified by comparison with the retention times of known standards, and identification was confirmed by selective coinjection of samples with standards.

Statistical analysis. χ^2 analysis was used to compare proportions of deoxyribose-positive samples between study groups. Logistic regression in univariate and multivariate analyses was used to determine odds ratios and their 95% confidence intervals for associations between the presence of the *deoK* operon and other variables (sex, age group, geographic origin, and clinical condition). *P* values of less than 0.05 were considered significant. A χ^2 test for trend was used to compare the proportion of deoxyribose-positive samples by the number of ExPEC virulence factors. Stata 8 (Stata statistical software; Stata Corporation, TX) was used for all statistical analyses.

The mean log CFU/g of feces for a particular *E. coli* strain was calculated at each time point in coinfection experiments A paired one-tailed Student's *t* test (GraphPad InStat software) was used to analyze differences. *P* values of less than 0.05 were considered significant.

RESULTS

Deoxyribose catabolism in the *E. coli* species. In order to obtain more information about the distribution of the metabolic trait of deoxyribose catabolism in the *E. coli* species, we characterized the ECOR collection for deoxyribose use. This collection includes 72 strains from diverse sites, hosts, and clinical contexts, selected to represent the breadth of genetic diversity within the species *E. coli* (43). One-third (33.33%) of the ECOR strains carried the *deoK* gene and were able to use deoxyribose as a carbon source, suggesting that they carry the entire *deoK* operon (data not shown). No significant differences in deoxyribose status frequency were observed between strains of animal or human origin (34.4% versus 32.5%, respectively) or strains from different human clinical specimens (normal stools from healthy hosts or urine from women with UTI; 30% versus 40%, respectively). However, the ECOR collection is mainly composed of commensal isolates (32 animal and 30 human commensal strains and 10 uropathogenic human strains), so the absence of difference in the frequency of deoxyribose-positive isolates between pathogenic and commensal human isolates is not relevant. We focused on the human commensal strains ECOR51, ECOR53, ECOR54, ECOR56, and ECOR63, which have been reported to belong to the B2 phylogenetic group and to carry more than two ExPEC virulence genes (30). Consequently, they may be considered to be uropathogenic ExPEC isolates residing in the intestine (17, 30). All five were deoxyribose positive. Thus, 60% (9/15) of the human uropathogenic (or potentially uropathogenic) strains of the ECOR collection, but only 16% (4/25) of the nonpathogenic strains isolated from the feces of healthy human subjects, were deoxyribose positive. These findings are consistent with an association between uropathogenic potential and deoxyribose catabolism in *E. coli* strains, as suggested in a previous study (5).

Prevalence of and factors associated with the *deoK* operon. In order to obtain more information about the distribution of this metabolic trait among human *E. coli* isolates, we further investigated *E. coli* isolates from 213 individuals from Romania, Senegal, and the CAR collected by laboratories of the International Network of Pasteur Institutes. We tested these strains both for growth on minimal medium containing deoxyribose and for the presence of the *deoK* gene. Most UTI involve only a single strain of uropathogenic *E. coli* (19), so only one colony was studied for each case of UTI. Urine samples were considered deoxyribose positive if the *E. coli* clone analyzed was positive. Because *E. coli* is a normal resident of the human intestinal microbiota, at least five colonies are usually studied to characterize *E. coli* in stool samples. Consequently, for stool samples (from healthy patients and patients with diarrhea), the median number of clones analyzed per sample was 7 (range, 3 to 20). Stool samples were considered deoxyribose positive if at least 60% of the *E. coli* isolates tested were positive. We also included data for 195 ExPEC strains isolated in France (5) in the study. Overall, 33.6% (386/1,149) of the clones were deoxyribose positive, a percentage similar to that found for the ECOR strains.

TABLE 3. Demographic and clinical factors associated with deoxyribose positivity

Factor	No. (%) of samples (n = 408)	Deoxyribose-positive samples[a] (n = 160)	OR (95% confidence interval)[b]	
			Univariate	Multivariate
Age group (yr)				
<15	58 (14.2)	21 (36.2)	1	1
15–45	98 (24.0)	36 (36.7)	1.02 (0.52–2.01)	1.12 (0.52–2.39)
>45	178 (43.6)	75 (42.1)	1.28 (0.70–2.37)	1.32 (0.62–2.82)
Missing	74 (18.1)	28 (37.8)	1.07 (0.53–2.19)	0.56 (0.19–1.64)
Geographic origin				
Europe	305 (74.8)	117 (38.4)	1	1
Africa	103 (25.2)	43 (41.8)	1.15 (0.73–1.81)	**4.20** (1.59–11.1)
Clinical condition				
Healthy	62 (15.2)	13 (21.0)	1	1
ABU[c]	11 (2.7)	2 (18.2)	0.84 (0.16–4.36)	**2.63** (0.41–16.6)
Cystitis	82 (20.1)	31 (37.8)	**2.29** (1.07–4.88)	**5.99** (2.11–17.0)
Pyelonephritis	112 (27.5)	55 (49.1)	**3.64** (1.78–7.43)	**23.1** (6.82–78.2)
Bacteremia	105 (25.7)	42 (40.0)	**2.51** (1.22–5.19)	**7.20** (2.31–22.5)
Diarrhea	36 (8.8)	17 (47.2)	**3.37** (1.38–8.26)	**2.79** (1.10–7.10)

[a] Percent of samples positive for deoxyribose metabolism.
[b] Odds ratio (OR) values significantly different from 1 are shown in boldface.
[c] ABU, asymptomatic bacteriuria.

A statistical analysis was performed to investigate the association of deoxyribose catabolism and other bacterial factors or clinical conditions. In total, 305 subjects (74.8%) were living in Europe when the strains were isolated; the other subjects lived in Africa. Most European participants were female (61.3%), over 45 years old (52.5%), and suffering from extraintestinal infections (60.3% with UTI and 34.4% with bacteremia). Most African subjects were under 45 years old (89.6%), and most either were suffering from diarrhea (35.0%) or were healthy (44.7%). One hundred sixty samples (39.2%) were deoxyribose positive. Univariate analysis indicated that symptomatic clinical conditions were associated with a greater risk of deoxyribose metabolism by the strain (Table 3). This association remained significant in multivariate analysis. African origin became significantly associated with deoxyribose catabolism in multivariate analysis after other variables were controlled for.

We used PCR assays to detect genes encoding five established ExPEC virulence factors (P fimbriae, S and Afa adhesins, hemolysin, and cytotoxic necrotizing factor 1), as previously described (36, 37, 46) (data not shown). We then tested for an association between ExPEC virulence potential and deoxyribose catabolism. For this analysis, multiple bacterial isolates from the same set of stool samples were considered independent; thus, data from a total of 610 clones (308 from normal stools, 197 from urine, and 105 from blood) were compared. The proportion of deoxyribose-positive strains in-

creased with the number of ExPEC virulence factors (32.0, 40.5, 41.9, 59.5, 70.4, and 100% for zero, one, two, three, four, and five virulence factors, respectively; test for trend, $P < 0.001$). However, among clones from normal stools, the frequency of deoxyribose catabolism was significantly higher for those that could be considered potential ExPEC strains (carrying more than two ExPEC virulence factors) than for those carrying no or one ExPEC virulence factor (93.7% [15/17] versus 34.6% [101/292]; $P < 0.001$). Thus, there is clearly an association between extraintestinal pathogenicity potential and deoxyribose catabolism in *E. coli* strains.

Role of deoxyribose catabolism in colonization of the host intestine. Colonization of the intestine is a necessary preliminary step for the development of either intestinal or extraintestinal *E. coli* infections. According to Freter's nutrient niche theory, implantation of an invading bacterium into the local microflora depends, in part, on its ability to outcompete the bacteria of the microflora for the metabolic use of the nutrients present (20, 21). Sugar metabolism has been shown to be very important for intestinal colonization by *E. coli* K-12 (9), and commensal *E. coli* isolates use deoxyribose significantly less frequently than pathogenic isolates. Therefore, expression of this metabolic trait may confer an evolutionary fitness advantage on pathogenic *E. coli*, enabling the invading strain to outcompete the indigenous microflora and colonize the intestine. Conventional mice display natural resistance to colonization of the gut by *E. coli* (21), and germ-free animals have no indigenous microflora, greatly affecting nutrient availability to the invading strain, particularly for limiting nutrients. We therefore investigated the role of deoxyribose catabolism in the streptomycin-treated-mouse model of intestinal colonization. In this model, the streptomycin treatment eliminates the facultative bacteria from the intestine, thereby opening a niche for *E. coli*, but leaves the anaerobic microbiota largely intact. Consequently, the *E. coli* bacteria fed to the mice face competition from a large commensal microbiota.

Before beginning animal experiments assessing the relative fitness of *deoK* mutants for colonization of the intestine with respect to their parental strains, we evaluated the levels of free deoxyribose among all common monosaccharides in mouse cecal mucus. Free monosaccharides were eluted from the mucus of different animals in identical volumes of water and were individually quantified after separation by HPAEC. The results for all the monosaccharides tested are shown in Fig. 1. The results of three independent determinations showed that free deoxyribose was present at limiting concentrations within each cecal-content sample. Cecal-content eluates contained about 2 μg/ml of free deoxyribose (2.2, 1.9, and 2.2 μg/ml), for a total concentration of 22 to 72 μg/ml of identified free monosaccharides (Fig. 1).

Role of *deoK* in colonization of the mouse intestine by the diarrhea-associated enteroaggregative *E. coli* strain 55989. A previous study showed that expression of the *deoK* operon by the enteroaggregative *E. coli* strain 55989 in vitro increases its competitiveness (5). We replaced the *deoK* gene in a streptomycin-resistant derivative of the 55989 isolate with a kanamycin cassette. As expected, strain 55989Str outcompeted its *deoK* mutant in cocultures in K5 minimal medium supplemented with pyruvate (0.4%) and deoxyribose (data not shown). We fed 10^5 CFU of 55989Str and 10^5 CFU of its *deoK* mutant independently to mice, and the two strains colonized the mouse intestine to similar levels (between 10^8 and 10^9 CFU/g of feces) 1 day postinfection. Both strains persisted at these levels throughout the assay (18 days) (data not shown). We used coinfection assays to detect subtle differences between the colonization abilities of the parental and mutant strains (6). Mice were fed simultaneously with 10^5 CFU of both the 55989Str and 55989Str *deoK* strains. The counts of the two strains in feces after 24 h were similar (Fig. 2A and B). The 55989Str strain remained present at a concentration of 10^8 to 10^9 CFU/g of feces throughout the 18 days, whereas levels of the *deoK* mutant decreased over time (Fig. 2A). No clear difference in colonization was observed between the two strains during the first week after challenge. However, the numbers of CFU per gram of feces for the two strains were significantly different from 12 days after challenge ($P = 0.0453$) until the end of the experiment ($P = 0.0238$ and $P = 0.0216$ on days 15 and 18 postinfection, respectively) (Fig. 2A). Similarly, the mean log CI was significant (less than -0.3) from 12 days after inoculation on (-0.78, -1.09, and -1.09 on days 12, 15, and 18 postinfection, respectively) (Fig. 2B). Therefore, although the *deoK* mutant persisted in the intestine when mice were fed this strain alone, it did not grow well enough to maintain itself in the intestine when fed to mice simultaneously with the 55989Str strain. We transcomplemented the 55989Str *deoK* mutant with pILL1314, a pUC18 derivative expressing the *deoK* gene from 55989 (5). The resulting strain grew on K5 minimal medium containing deoxyribose as the sole source of carbon and energy. Consistent with previously reported difficulties in obtaining phenotypic complementation in animal models (29, 1), we were unable to demonstrate complementation of the *deoK* mutation by pILL1314 in the mouse colonization model. This led us to develop a different approach to demonstrate that the deletion of *deoK*, and not expression of the kanamycin cassette, was responsible for the decrease in competitiveness of the *deoK* mutant in coinfections. We investigated the role of mutations, obtained by the same protocol of replacement with a kanamycin resistance cassette, in genes involved in different metabolic pathways. We constructed mu-

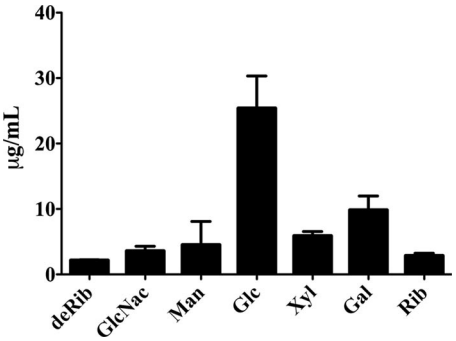

FIG. 1. Concentration of free monosaccharides within the cecal contents. Monosaccharides were collected from the mucus by thorough rinsing, and their individual concentrations were established from three independent samples by HPAEC on a carbopack MA1 column. Concentrations are given in μg/ml of cecal eluates. deRib, deoxyribose; GlcNAc, *N*-acetyl-glucosamine; Man, mannose; Glc, glucose; Xyl, xylose; Gal, galactose; Rib, ribose. The error bars indicate standard deviations.

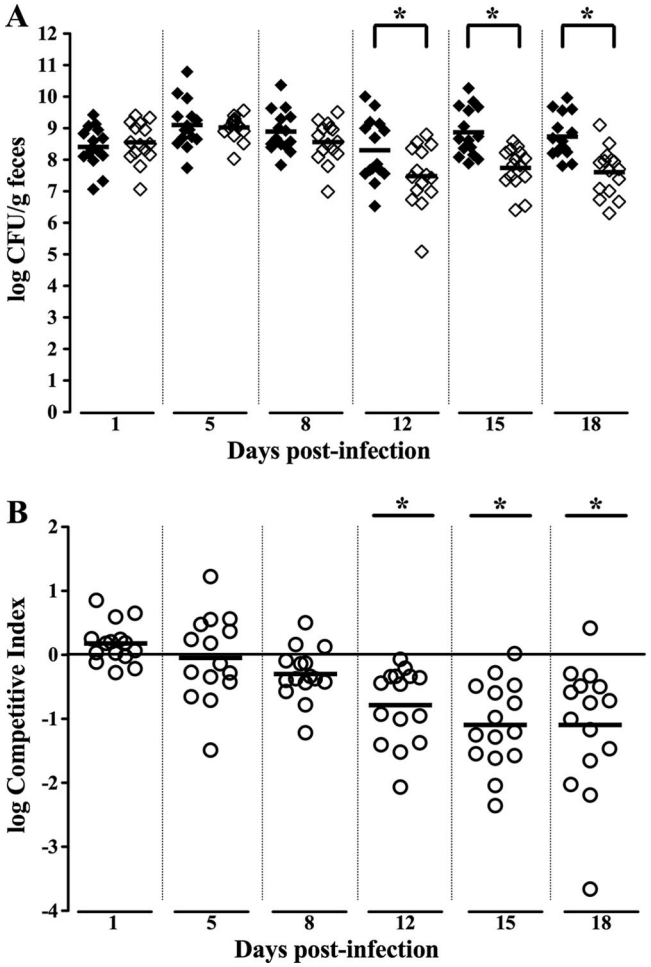

FIG. 2. Competitive colonization of the mouse intestine by 55989Str and 55989Str *deoK*. 55989Str and 55989Str *deoK* were administered simultaneously (1:1 ratio) by oral gavage to streptomycin-treated BALB/c mice. Two independent colonization experiments involving a total of 15 mice were performed. At the indicated times, fecal samples were homogenized, diluted, and plated on LB agar with or without kanamycin. (A) The results are reported as log CFU/g feces for 55989Str (◆) and 55989Str *deoK* (◇). The horizontal bars represent the mean values. The brackets with asterisks indicate that *P* was <0.05 in a paired one-tailed *t* test. (B) The results of the experiments are reported as the log of the CI. The CI was calculated as the ratio of mutant to wild-type CFU recovered from mice at the various times divided by the initial mutant-to-wild-type CFU ratio. The line at a *y* value of 0 corresponds to the expected log CI if there were no difference in the capacities of the two strains to colonize and persist in the mouse intestine. Negative values for log CI indicate a competitive advantage for the wild-type strain over the mutant. Each point (○) corresponds to a single mouse, and the horizontal bars represent the mean values. The asterisks indicate mean values of less than −0.3, reflecting significant attenuation of the mutant. The *deoK* mutant was significantly outcompeted by the wild type from day 12 postinfection.

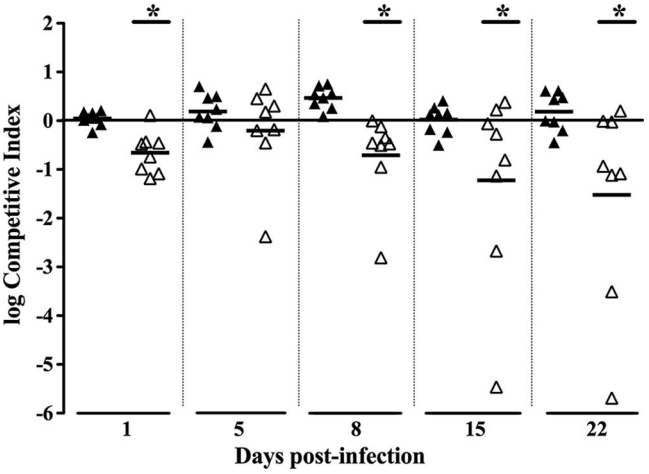

FIG. 3. Competitive colonization of the mouse intestine by 55989Str and metabolic mutants. Two independent colonization experiments were performed with a total of eight mice. Sets of three and five mice were simultaneously fed with 55989Str and 55989Str *glpK* (▲) or 55989Str and 55989Str *edd* (△). The results are reported as the log CI. In the calculation of CI, we arbitrarily attributed a value of 10^1 CFU/g of feces to a strain if no bacteria were recovered on plates (detection limit, $<10^2$ CFU/g of feces). Each point corresponds to a single mouse, and the horizontal bars represent the mean values. The asterisks indicate mean values of less than −0.3, reflecting significant attenuation of the mutant. The *edd* mutant competed very poorly with the wild type at all time points, even if at 5 days postinfection the mean log CI was below the significance threshold (log CI = −0.27). No competition was observed between the *glpK* mutant and the wild type.

tants with defects in gluconate and glycerol catabolism: gluconate catabolism contributes to the colonization of the mouse intestine by *E. coli* K-12, whereas glycerol catabolism does not (9). We carried out coinfections of mice with the 55989Str *edd* (gluconate catabolism) and 55989Str *glpK* (glycerol catabolism) mutants and the parental strain. The 55989Str parental strain significantly outcompeted the *edd* mutant during both the initiation (log CI, −0.56 1 day postinfection) and the maintenance (log CI, less than −1.52 22 days postinfection) stages of colonization (Fig. 3). The *glpK* mutant and its parental strain displayed similar levels of colonization of the intestine and persistence throughout the 22 days of the experiment (Fig. 3). The *glpK* gene thus does not contribute to colonization. Our results are consistent with those reported for *E. coli* K-12 mutants obtained with a different mutagenesis protocol (9). These data clearly demonstrated that the intestinal-colonization phenotype depended on the gene into which the cassette was inserted. The colonization defect of the *deoK* mutant was therefore not associated with the kanamycin resistance cassette. Consequently, our findings indicate that metabolic competition for deoxyribose plays a role in maintaining the colonization of the mouse intestine by strain 55989Str.

Role of *deoK* in colonization of the mouse intestine with the pyelonephritis-associated *E. coli* strain AL511. We investigated whether the colonization defect associated with a *deoK* deletion was restricted to strain 55989Str or applied to other *E. coli* clinical isolates. We tested strain AL511, a naturally streptomycin-resistant pyelonephritic isolate, in the mouse model of colonization. Like *E. coli* 55989Str, AL511 colonized the mouse intestine following inoculation of 10^7 CFU. The level of colonization 1 day after inoculation was variable, but the bacterial count increased to and stabilized at 10^8 to 10^9 CFU/g of feces during maintenance in the intestine (day 5 to day 18) (data not shown). AL511 *deoK* mutants were constructed with a kanamycin cassette different from that used to obtain 55989Str *deoK*, providing further evidence that the antibiotic cassette used to replace the *deoK* gene had no cost in terms of coloni-

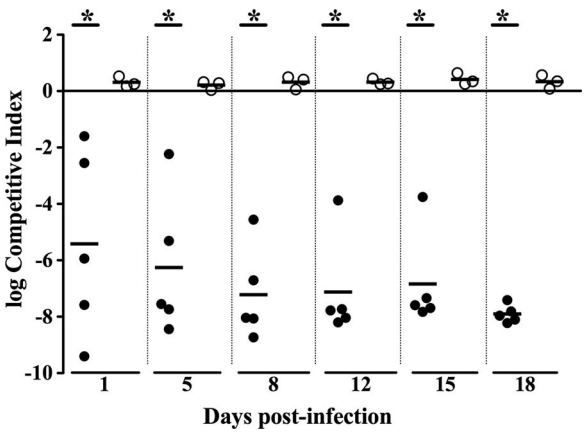

FIG. 4. Colonization of the mouse intestine by AL511 and AL511 *deoK* derivatives. Sets of five mice were simultaneously fed with either the wild-type strain and AL511 *deoK* (●) or the two *deoK* derivatives AL511 *deoK* (Km^s) and AL511 *deoK* (○). For competition between the two derivative strains, only three out of five mice were colonized. The results are reported as log CIs: AL511 *deoK*/wild-type and AL511 *deoK*/AL511 *deoK* (Km^s) ratios at each time point divided by the corresponding ratio at the initial point. In the calculation of the CI, we arbitrarily attributed a value of 10^1 CFU/g of feces to a strain if no bacteria were recovered on plates (detection limit, $<10^2$ CFU/g of feces). Each point represents a single mouse, and the horizontal bars represent the mean values. The asterisks indicate mean values of less than −0.3, reflecting significant attenuation of the strain. The *deoK* mutant was outcompeted by the wild type, whereas no competition was observed between the two *deoK* mutants, indicating that the kanamycin cassette had no effect on intestinal colonization by AL511.

zation ability. We first constructed an AL511 derivative in which the *deoK* gene was replaced by the FLP recombination target (FTR)-flanked kanamycin cassette (16). We then obtained the AL511 *deoK* (Km^s) mutant, in which the kanamycin cassette was deleted through the action of flipase on the FRT sites (Table 1). We verified that AL511 *deoK* mutants were unable to use deoxyribose; this deficiency was complemented by introducing the *deoK* gene (on pZEZeo*deoK*) in *trans*. As a control, the presence of pZEZeo*GFP* in the AL511 and *deoK* mutant strains did not affect deoxyribose use by the strains.

In the mouse colonization model, AL511 *deoK* and AL511 *deoK* (Km^s) when given alone colonized the intestine, reaching levels of 10^8 to 10^9 CFU/g of feces during the maintenance stage of the colonization process (data not shown). Next, mice were fed AL511 *deoK* along with either the parental isolate or the AL511 *deoK* (Km^s) strain. As for the 55989Str strain, AL511 colonized the intestine more efficiently than AL511 *deoK* (Fig. 4). Indeed, AL511 *deoK* counts fell earlier and faster than did 55989Str *deoK* counts in competition with their parental strains: its counts started to fall within 1 day of inoculation (mean log CI, −5.41), and it was totally eliminated from the feces within 22 days (data not shown). Assuming that no cost could be attributed to the kanamycin cassette introduced in place of the *deoK* gene in strain AL511 *deoK*, we expected AL511 *deoK* and its kanamycin-sensitive derivative [AL511 *deoK* (Km^s)] to have similar colonizing abilities. Mice were fed with the two mutant strains simultaneously, and both strains increased in number to 10^8 CFU/g of feces 1 day following infection; colonization was maintained at this level throughout the 22 days of the experiment (data not shown). The mean log CI remained close to zero throughout the time course (Fig. 4). This confirmed that the kanamycin cassette used had no significant influence on the results.

DISCUSSION

The gut microflora constitutes a highly competitive environment, and thus a barrier to colonization, decreasing the probability of ingested pathogenic strains establishing themselves in the intestinal ecosystem of a healthy host (27). Pathogenic *E. coli* infections in humans and animals are transmitted by the orofecal route. Adaptation to the intestinal environment is therefore the first step in infection by all pathogenic *E. coli* strains (33). It remains unclear how pathogenic *E. coli* strains achieve this and whether the catabolism of particular nutrients is involved in outcompetition of the commensal *E. coli* strains by pathogenic strains. We previously reported that the *deoK* operon, which encodes proteins required for the use of deoxyribose as a carbon source, is more prevalent in intestinal and extraintestinal pathogenic than commensal *E. coli* strains (5). We also showed that the expression of the operon increases the competitiveness of clinical isolates, implicating this biochemical trait in bacterial infectivity (5). Here, we have shown that deoxyribose is present in the mouse cecal contents. We then investigated the potential role of deoxyribose catabolism in colonization of the mammalian intestine by pathogenic *E. coli* isolates.

We initially determined the frequency and distribution of the *deoK* operon in the *E. coli* species. We investigated a total of 1,221 *E. coli* isolates from various collections at both the molecular and biochemical levels. Overall, 33.6% of the isolates were deoxyribose positive, indicating that the *deoK* operon has been stably maintained in *E. coli* since its horizontal transfer from *Salmonella enterica* (5). Isolates from individuals with symptomatic clinical conditions were significantly associated with increased risk of deoxyribose positivity. The genome sequences of several deoxyribose-positive pathogenic isolates are (strains CFT073, 536, and O42) or will soon be (strain 55989; ColiScope Consortium) available from databases. The *deoK* operon has been found to be located in a large specific island carrying genes contributing to the intrinsic virulence and/or adaptive properties of the strain.

An African origin of the biological sample became significantly associated with deoxyribose positivity in multivariate analysis after other variables were controlled for. There are two possible explanations for this. Intestinal diseases are endemic in Senegal and the CAR, and typhoidal *S. enterica* is frequently involved; this may result in a high frequency of genetic transfer between *Salmonella* and *E. coli*. Alternatively, the frequent destruction of the intestinal epithelia by pathogens may generate additional deoxyribose in the lumen, leading to positive selection for deoxyribose metabolism-positive *E. coli* in the microflora. Among strains isolated from stools from the two African countries, deoxyribose metabolism was significantly associated with diarrhea. *E. coli* was isolated as an almost pure culture on *Enterobacteriaceae* selective media. We therefore suspected that *E. coli* was the true etiological agent of these enteric syndromes. However, genotypic analyses failed to classify the clones present into the six well-characterized diarrheagenic pathotypes of *E. coli*. It therefore appears that

E. coli clones capable of metabolizing deoxyribose isolated from diarrheagenic stools are opportunistic agents exploiting their ability to metabolize a sugar whose concentration in the intestinal contents is increased by the diarrhea process. Consequently, these clones become predominant in the intestinal facultative-anaerobic microflora.

Nevertheless, standard in vitro adhesion assays (42) indicated that all the clones tested (4 to 10) from four stool samples from patients with diarrhea in Senegal had an aggregative adhesion phenotype specific to the EAEC pathotype (data not shown). The EAEC pathotype is an emerging cause of diarrhea worldwide (28). All the clones in three of these stool samples were also deoxyribose positive. This is consistent with previous studies identifying EAEC as an etiologic agent of diarrhea in Senegal (22) and those showing a high frequency of deoxyribose positivity in EAEC strains (55.5%) (5). We therefore investigated the role of deoxyribose in intestinal colonization with the EAEC isolate 55989, using the streptomycin-treated-mouse model of intestinal colonization. This model has been used extensively, in particular, to demonstrate that the initiation and maintenance stages of mouse gut colonization by *E. coli* K-12 strain MG1655—a human commensal (9) competing with microflora—is influenced by the catabolism of several limiting monosaccharides in the cecum. We knocked out the pathway responsible for deoxyribose metabolism and showed that the parental isolate 55989Str outcompeted its *deoK* derivatives during the maintenance stage of mouse gut colonization. During this stage, persistence has been reported to depend on nutrients that become limiting (9). Consequently, our findings indicate that expression of the *deoK* operon by *E. coli* 55989 is involved in the persistence of this clinical isolate in the intestine.

We confirmed that a large proportion of ExPEC strains isolated from blood and urine are deoxyribose positive. This finding was supported by studies of the pathogenic potentials of ExPEC isolates involving detection of virulence factors. The presence of the *deoK* operon was significantly associated with the presence of virulence factors. Also, most (>93%) of the potential ExPEC strains resident in the bowels of healthy subjects were deoxyribose positive. ExPEC strains have been reported to colonize the intestine stably without causing clinical symptoms and are the predominant *E. coli* strains in approximately 20% of healthy individuals (44). We then investigated the role of deoxyribose metabolism in colonization by strains of this group. We evaluated the gut colonization potential of the uropathogenic isolate AL511 in the streptomycin-treated-mouse model. In particular, we tested whether intestinal colonization by an ExPEC strain depended on expression of the *deoK* operon by evaluating the relative fitness of the parental strain and *deoK* mutants in coinfection experiments. As for strain 55989Str, disruption of the *deoK* operon led the mutant to be outcompeted by the wild type. However, this effect occurred earlier and was stronger than with the EAEC strain (mean log CI, −5.41 1 day postinfection for AL511 versus −1.09 18 days postinfection for 55989Str). It was first observed during the initiation stage of gut colonization, which has been reported to depend on the use of nonlimiting nutrients (9). These experiments demonstrated a role for deoxyribose catabolism in gut colonization, and presumably therefore infection, by *E. coli* isolates of two different pathotypes in vivo. Nevertheless, not all isolates seemed to use deoxyribose in the same way. Analysis of other pathogenic isolates should reveal whether differences in colonization behavior are associated with differences in the regulation of the *deoK* operon expression or differences in the specific metabolome or the pathotype.

Many virulence factors affecting a wide variety of host cell processes at the site of infection have been identified. However, it remains unclear how pathogenic *E. coli* strains acquire the nutrients required for host infection during colonization of the gastrointestinal tract before initiation of the disease process at various infection sites. We report the first example of sugar catabolism providing a nutritional basis for cocolonization of the gut with the commensal *E. coli* flora. The Nissle 1917 strain, a successful colonizer of the human gut, is able to ferment deoxyribose. This strain, which has the characteristics of uropathogenic *E. coli* but is completely nonpathogenic (25), has been used for decades in human medicine in Central Europe (48), particularly for the intentional colonization of the gut in newborn infants to prevent the acquisition of pathogenic bacteria (38). Deoxyribose fermentation may be one of the ways in which this strain acts as a probiotic, facilitating its own persistence in the gut and preventing colonization of the intestine by pathogenic *E. coli* strains that compete poorly with it for limiting nutrients. Comparative genomic studies with more than 50 *E. coli* strains for which genome sequences have been completed or are in progress (http://www.genomesonline.org/gold.cgi/) should facilitate the identification of such biochemical characteristics and increase our understanding of how pathogenic *E. coli* strains colonize the intestine and persist in the fecal flora.

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